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Regulation, evolution and consequences of cotranslational protein complex assembly

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Most proteins assemble into complexes, which are involved in almost all cellular processes. Thus it is crucial for cell viability that mechanisms for correct assembly exist. The timing of assembly plays a key role in determining the fate of the protein: if the protein is allowed to diffuse into the crowded cellular milieu, it runs the risk of forming non-specific interactions, potentially leading to aggregation or other deleterious outcomes. It is therefore expected that strong regulatory mechanisms should exist to ensure efficient assembly. In this review we discuss the cotranslational assembly of protein complexes and discuss how it occurs, ways in which it is regulated, potential disadvantages of cotranslational interactions between proteins and the implications for the inheritance of dominant-negative genetic disorders.

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Introduction

Many proteins can assemble into protein complexes [1,2•]. Although there is tremendous diversity in the types of quaternary structures that can be formed [3,4••], at the simplest level, protein complexes belong to two categories: homomers, formed from multiple copies of the same protein subunit, and heteromers, which have at least two distinct subunits with different amino-acid sequences. While homomers and heteromers are both prevalent across evolution, most prokaryotic complexes are homomers, while most eukaryotic complexes are heteromers [5–7].

Protein complexes are crucial for a large number of biological functions, and different types of protein quaternary structures have been shown to facilitate different biological functions and allosteric regulation [8•,9–12]. A large number of other benefits have been proposed [4••,13]. For example, considering the possibility of acquiring mutations during transcription and translation, it is more efficient to synthesize a larger structure in modules of subunits. Importantly, it also allows fine spatial and temporal regulation, and reduces folding complexity in forming unique shapes such rings or filaments. It has also been shown that multiple identical domains of the same polypeptide chain are prone to aggregation [14] due to formation of domain-swapped structures during cotranslational folding [15•]. Therefore, translating these domains as separate polypeptides that later assemble into a large complex can be less risky. Finally, it is important to emphasize that, while clearly there are many advantages to protein complexes, protein oligomerization is not always functionally beneficial and the result of evolutionary selection, but may be explained by simple nonadaptive processes [6,16].

In recent years, we have learned a considerable amount about the processes by which proteins assemble into complexes. We know that proteins generally assemble via ordered pathways that tend to be evolutionarily conserved [17,18]. Moreover, these assembly pathways appear to be biologically important both in prokaryotes [19] and eukaryotes [20]. However, there are still unanswered questions about how the cell regulates protein complex assembly, and where assembly actually occurs within the cell. A logical place to begin addressing this is in the initial stages of protein synthesis and folding.

Cotranslational folding and assembly

The phenomenon of cotranslational folding has received considerable attention in recent years. Although the exact frequency at which cotranslational folding occurs in either prokaryotes or eukaryotes is unknown, there is a large body of computational [21–23] and experimental work [24,25••,26,27••] supporting and defining its likelihood. Significantly, these works emphasize the balance between the rate of translation, for example, as a function of charged-tRNA availability [28] or mRNA secondary structure [29–31], and the rate of protein folding. For reviews on the topic we recommend [32–34].

There are several reasons why proteins might acquire secondary structure during translation, sometimes even while still inside the ribosome exit tunnel [24,35,36]. For example, folding cotranslationally can modify the potential energy landscape to avoid nonproductive intermediates that would prevent the protein from reaching its native state [28]. However, cotranslational folding also reduces the propensity of deleterious non-specific interactions with the crowded cellular milieu or with other polypeptides on the same polyribosome. In other words, the protein primarily folds to protect itself from nonspecific interactions, but in doing so also allows assembly with native partners.

Given the prevalence of cotranslational folding, it is natural to imagine that assembly could also occur cotranslationally, especially given that folding and assembly are so intimately related [37]. This could potentially be beneficial for many of the same reasons as cotranslational folding; in particular, it could protect the protein from non-specific interactions, which is crucial due to the presence of the exposed interfaces making the unassembled subunits very sensitive to aggregation. This is particularly true for soluble homomers, which typically form larger hydrophobic interfaces than heteromers, and are thus more prone to misinteraction [38]. Although cotranslational assembly has received far less attention than cotranslational folding, it has been known of for a long time, with the first example we are aware of being homotetrameric β-galactosidase published in 1964 [39]. More recently, evidence is emerging that the phenomenon may be widespread [34,40••].

How does cotranslational assembly occur within the cell?

During cotranslational assembly, at least one of the protein subunits begins to assemble while it is still in the process of being translated, that is, the interaction involves a nascent chain. This can occur via either *cis* or *trans* mechanisms. The *cis* mechanism (Figure 1a) involves the assembly of polypeptides from the same mRNA; this can refer either to the case where an interaction occurs while both chains are still in the process of being translated, or when a nascent chain binds to a fully translated protein released by the same mRNA. In contrast, the *trans* mechanism (Figure 1b) involves the assembly of a polypeptide from one mRNA with the product of another, and can apply to either heteromeric or homomeric assembly.

The rate at which cotranslational assembly will occur is a function of the affinity of the subunits for one another, and their effective concentration. However, concentration in this case is not purely determined by the number of proteins in solution, but also by the density of nascent polypeptides on the polyribosome. An important parameter influencing this is the length of time a nascent polypeptide spends attached to the mRNA, which in turn depends on numerous factors, including mRNA secondary structure [30], the availability of charged-tRNAs, the overall length of the mRNA, and elements such as anti-Shine-Dalgarno sequences in mRNA [41]. Thus, concentration is a function of multiple variables, but for simplicity can be summarized as the total number of nascent polypeptides within the polyribosome's sphere of influence at a particular point in time.

At this point, we would like to propose an additional role to the secondary structure of mRNA. As mentioned above, the secondary structure of mRNA affects translation rate, thus regulating nascent chain folding into its correct fold. However, it is likely that many mRNAs form more complex structures than that of the two-dimensional structure, and thus the polyribosome and consequently the ribosome tunnels will be orientated in a particular way. These trajectories will influence both the probability of clashing between nascent chains, which will affect the stability of monomers, and the probability of cotranslational complex assembly. It is therefore important to understand the native three-dimensional of the polyribosome, continuing recent efforts [42••,43•].

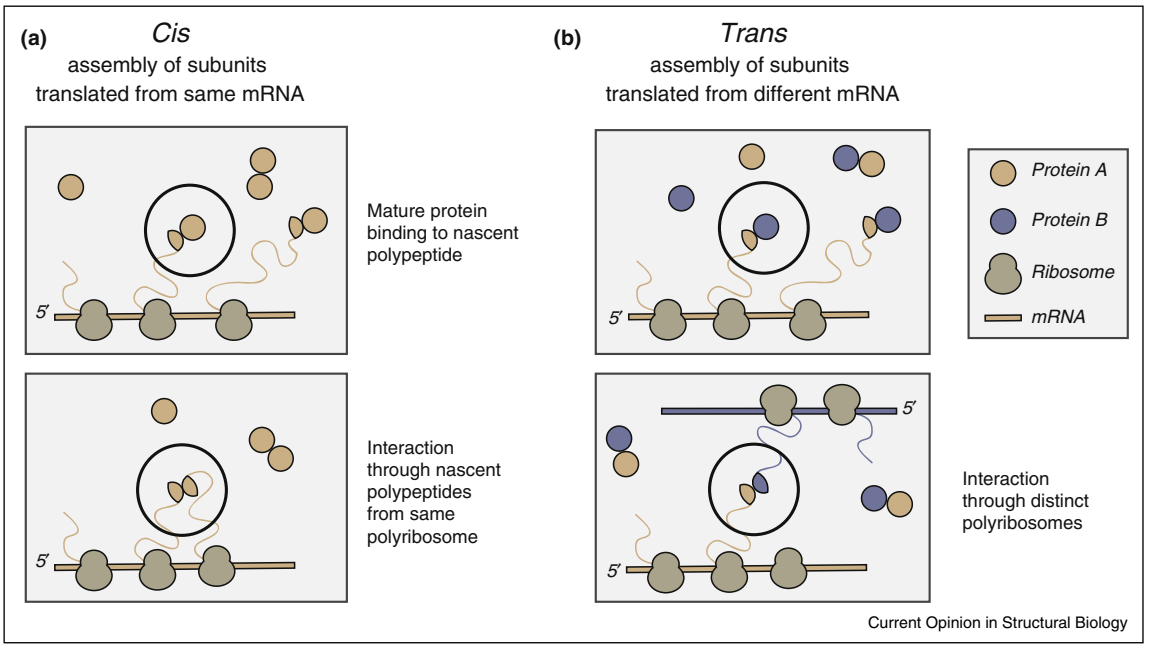
The cell broadly regulates both *cis* and *trans* mechanisms. For *cis*, the number of ribosomes, which is a function of 'initiation rate' (how many), 'elongation' (how long), and 'termination', will determine its frequency of occurrence. For the *trans* mechanism, concentration can be increased by active transport of the same-gene mRNAs transcripts to a specific location in the cell, a mechanism which has been observed in both eukaryotes [44] and prokaryotes [45,46]. It is worth mentioning that this factor is rarely discussed in the literature, and should be taken into account while discussing mRNA localization of protein complexes.

Cotranslational assembly of operon-encoded complexes

At this juncture, it is important to highlight the stark differences between eukaryotic and prokaryotic assembly of protein complexes, specifically for heteromers. In eukaryotes, cotranslational assembly of heteromers must occur in *trans*, either through co-localization of mRNAs encoding interacting proteins, or through localization of fully folded proteins to active polysomes (Figure 1b). In contrast, prokaryotes often encode protein complex subunits in operons, whereby distinct protein subunits can be translated from the same polycistronic mRNA molecule [47,48]. Thus, for operon-encoded complexes, cotranslational assembly of heteromers can occur in *cis* in much the same way as it does for homomers (Figure 2).

To this end, there are multiple strands of evidence pointing to the important role operons play in facilitating

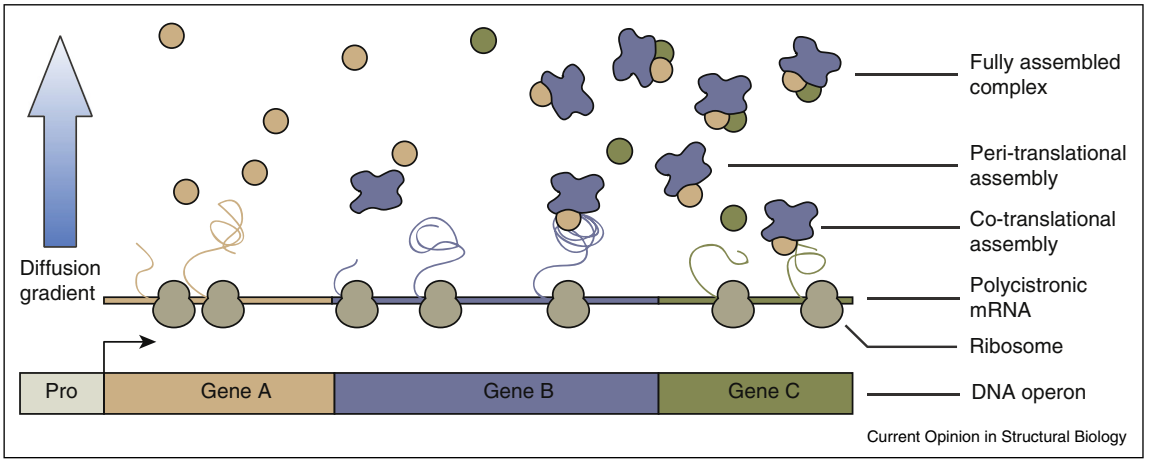
Figure 1



Cotranslational assembly can occur via **(a)** *cis* or **(b)** *trans* mechanisms, in which the interacting subunits are translated from either the same or different mRNA molecules. Moreover, either one or both subunits may still be in the process of being translated when the interaction occurs. *Cis* assembly exclusively involves homomers, whereas *trans* assembly can involve either two subunits encoded by the same gene (homomers) or different genes (heteromers).

complex assembly. In recent work using a modified luciferase system, Shieh *et al.* [25••] demonstrated that encoding the genes for LuxA and LuxB within a single operon leads to markedly improved assembly efficiency compared to encoding them in different operons. They were further able to show directly that interactions between LuxA and LuxB were being formed cotranslationally. This was achieved by co-purifying YFP-tagged subunits with ribosomes that were actively translating untagged partner proteins.

Figure 2



Prokaryotic heteromers are often encoded in operons, whereby multiple genes are transcribed onto a single polycistronic mRNA. The order of genes in operons is highly non-random, and has been selected for such that adjacent genes on the operon are more likely to physically interact as proteins. Similarly, the order in which the protein complex assembles typically reflects the order in which the genes are encoded. This implies that assembly occurs cotranslationally, facilitated by the close proximity of interacting subunits. Further support comes from the fact that the correlation between gene order and assembly order is stronger for weakly expressed complexes, where it is essential that assembly takes place rapidly, before subunits diffuse away from the site of translation.

A complementary approach to the experimental work just described used computational analysis of structural and genomic data to demonstrate a strong correspondence between operon gene order and the assembly order of protein complexes, that is, proteins that are translated first tend be those that assemble first [19]. Moreover, adjacent genes in operons are far more likely to encode physically interacting proteins that form large interfaces than those separated by intervening DNA. For our purposes, the important implication arising from this is that these subunits must be assembling cotranslationally or very shortly after translation (i.e. peri-translationally). If not, then any selection for gene order would be rendered effectively neutral due to the diffusion away from the site of translation occurring prior to assembly. These studies, along with reports of increased yield in protein complexes from using native operon order when designing expression vectors [49], make it clear that cotranslational assembly of heteromers must be widespread in prokaryotes.

Factors influencing cotranslational assembly and its influence on protein complex evolution

Clearly there are advantages to cotranslational assembly, such as misinteraction avoidance and speed of assembly, but are there any drawbacks that might limit its occurrence in nature? One such drawback was first demonstrated by Jaenicke and colleagues [50–53], who showed that *in vitro* refolding of homomeric proteins after denaturation is more challenging than it is for monomeric proteins, presumably due to premature assembly [54]. Here we highlight a few additional scenarios in which cotranslational assembly may have deleterious effects.

First, assembly may slow or even pause ribosomes from their rapid unidirectional sliding along mRNA [55]. Second, assembly constrains the freedom of the nascent chain to freely rotate in all three rotational axes in the quest for the native fold. Limiting the polypeptide's rotational freedom may in fact direct the protein to the correct fold, that is, by limiting undesirable folds, but that may not be the case for all proteins. For example, knotted proteins, unique topological structures that form via the thread of one terminus through a loop of an intermediate conformer [56], are likely to avoid cotranslational assembly. Last, *cis* cotranslational assembly may force high proximity between two (or more) unfolded nascent chains; in other words, upon assembly a triangle-like conformation is adopted by the chains, with the tip of the triangle being the assembly point connecting two partially unfolding nascent chains. This premature assembly scenario could also explain the *in vitro* work of Jaenicke and colleagues.

Following this line of work, we hypothesized that cotranslational assembly is likely to be limited by different constraints because of the unique situation cotranslational assembly forces upon the nascent chains; that is, the linking of these molecules in the midst dynamic elongation and folding processes. Therefore, we performed a combined computational and experimental analysis to investigate this phenomenon [57]. First, we observed highly significant trend for interface-forming residues in homomers to be located towards C termini across thousands of protein structures and diverse kingdoms of life. This was in contrast to heteromers, where no such tendency was observed. We suspect this trend is the result of cotranslational assembly being evolutionarily selected against under certain circumstances: localization of interfaces towards C-termini will reduce the chance of cotranslational assembly since interface-forming residues will be translated last. To address this further, we expressed all homomers of *Escherichia coli* with known structures and assessed them for their *in vivo* aggregation propensities. Interestingly, the results showed that homomers with N-terminal interfaces are more likely to show an early and severe aggregation, supporting the idea that cotranslational interactions between homomeric subunits can lead to protein misfolding and misassembly.

We also investigated the factors that allow successful cotranslational assembly by engineering a library of constructs comprising three components organized in different orders: first, oligomerization domain that folds cotranslationally, second, a linker, and third, reporter genes. The position of the oligomerization domain was critical for the stability of the protein: positioning it at the N terminus results in misassembly, which correlates with the propensity for assembly to occur cotranslationally, in comparison to the well-folded C-terminal variant. However, successful assembly can still occur via the N terminus if a linker extends the distance between the oligomerization domain and the reporter, which suggests that the increase of the linker could either decrease local concentration and thus the propensity to assemble. Alternatively, if cotranslational assembly did occur, the local concentration of the partially unfolded nascent chains is reduced, thus lowering the propensity for misassembly. Finally, increasing the reporter's folding rate also allows successful cotranslational assembly via the N terminus, suggesting that enhanced folding of a domain adjacent to the assembly site increases the probability for protein stability. This finding may also align with the notion of extreme proximity of nascent chains upon assembly, whereby acquiring secondary structures fast enough protects the polypeptide from non-specific interactions.

This is the first work to our knowledge to describe the parameters by which cotranslational assembly works. However, it mainly focused on mechanisms encoded in the protein primary sequence, such as the location of residues participating in assembly or protein folding rate. Clearly, other factors such as chaperones may participate in ensuring correct assembly both for homomers and heteromers.

The role of cotranslational chaperones in regulating assembly

Chaperones play an essential role in avoiding misfolding or aggregation, thus promoting the formation of native tertiary and quaternary protein structure. The mechanistic details of how they act vary dramatically, and chaperones as a whole encompass a wide variety of unrelated protein families. There are several chaperones that directly assist the assembly of protein complexes. For example: the PAC family, which form intra-family heterodimers that assist with the assembly of heptameric alpha-subunit rings in the proteasome [58].

However, chaperones more often facilitate assembly indirectly, by ensuring that unfolded proteins reach their native-state safely, thus allowing correct assembly later [59]. Proteins are most vulnerable to formation of non-specific interactions during the process of translation, and thus it is unsurprising that many of these chaperones themselves act cotranslationally. For example, Hsp70 family members, together with Hsp40 co-chaperones, can interact cotranslationally with nascent polypeptide chains, protecting them against premature misfolding and aggregation [60]. Similarly, TRiC and the prokaryotic Trigger factor act downstream, facilitating folding and oligomeric assembly [61].

The action of chaperones is particularly important for eukaryotic proteins, which are typically longer than those from prokaryotes, often comprise multiple domains, and have a higher incidence of intrinsically disordered and flexible regions [7,62,63], which is in stark comparison to prokaryote proteins that shift the folding process towards a posttranslational route ([60] and references therein). The implication for our discussion is that we should expect to find more examples of chaperone involvement, directly or indirectly, in cotranslational assembly.

A final intriguing case is that of cotranslational interaction between human mitochondrially encoded COX1 and C12ORF62 [64]. COX1 is the first subunit of cytochrome c oxidase (complex IV of the respiratory chain complexes). During translation by the mitochondrial ribosome, it associates cotranslationally with two membrane-embedded assembly factors: first C12ORF62 and then MITRAC12. This enables interaction with the nuclear-encoded COX4, which is the second complex IV subunit to bind. Crucially, COX4 is the trigger for the release of COX1 by the ribosome. In COX4-depleted cells, the nascent COX1-C12ORF62 intermediate is held in an 'assembly-primed' state and simply accumulates in the mitochondrial inner membrane. As a result, those mitochondrial ribosomes translating COX1 are prevented from creating further copies of COX1. The mechanistic details of this process are not yet fully understood, but it has a fascinating implication, namely that mitochondrial translation activity can react to changes in the production of nuclear-encoded proteins. When cytoplasmic production of complex IV subunits slows, so too does mitochondrial production, despite the fact that the subunits in question are encoded on different genomes.

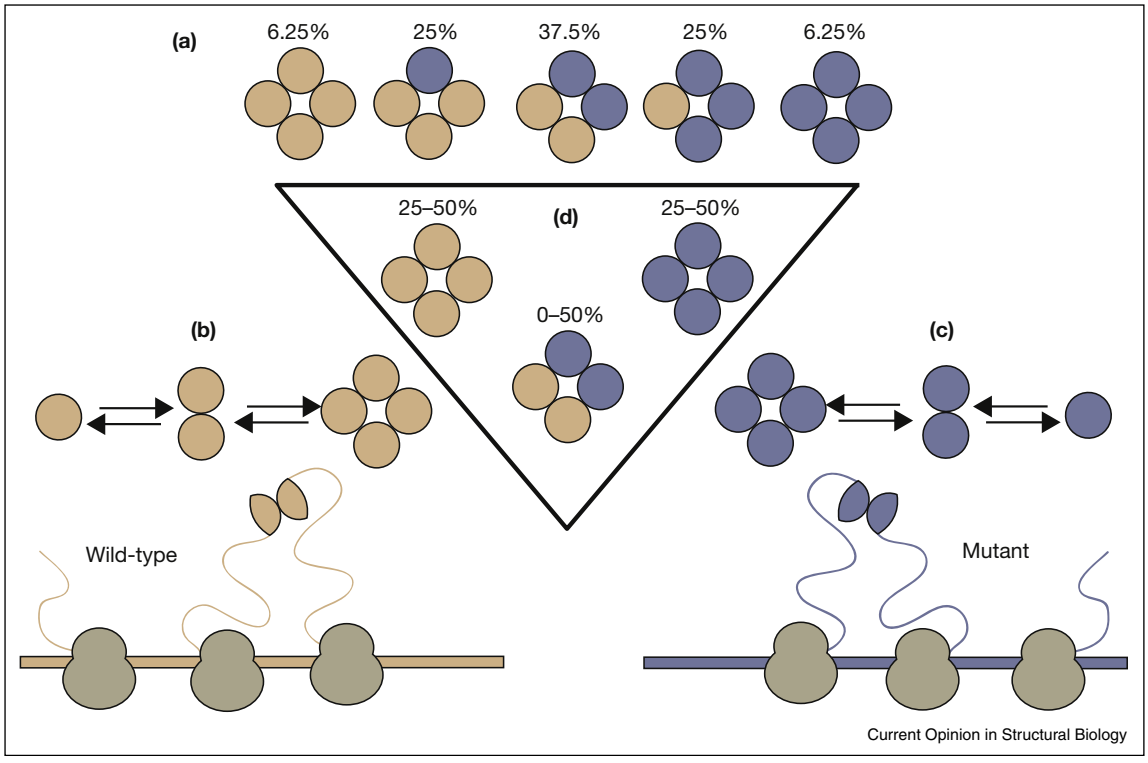
Implications of cotranslational assembly for the inheritance of genetic disease

The phenomenon of cotranslational assembly is not just important for understanding protein complex regulation and evolution: it also has potentially very important implications for genetic disorders associated with a dominant-negative (DN) mode of action. Essentially, a DN effect occurs when expression of a mutant allele can disrupt the activity of the wild type allele [65], thus resulting in a dominant mode of inheritance. DN effects have often been seen for genes that encode proteins that assemble into homomers [66]. The reason for this is simple: if the presence of a single mutant subunit within a complex is enough to 'poison' of the complex, the result will be a far greater reduction in activity than the 50% expected for a simple heterozygous loss-of-function mutation. In fact, DN mutations tend to be significantly less destabilizing towards protein structure than other pathogenic mutations because the mechanism requires that complex is still able to assemble [67].

To illustrate this, we can consider the case of a homotetramer encoded by a heterozygous allele. If both subunits are expressed at equal levels and associate randomly, then only 1/16 (6.25%) of the assembled complexes will be fully wild type homomers (Figure 3a). In contrast, if assembly occurs in *cis*, that is, cotranslationally or peri-translationally, the stoichiometry of the assembled complexes will be different. If all assembly occurs in *cis*, the homomeric products will be homogeneous, with half of the assembled complexes being fully wild type (Figure 3b) and half being fully mutant (Figure 3c). Finally, if not all of the second assembly step (dimerization of dimers) occurs peri-translationally, or there is equilibrium exchange between tetrameric and dimeric states, then the proportion of full wild-type complex will be smaller, but still greater than in the case of totally random assembly (Figure 3d). Therefore, the phenomenon of cotranslational assembly should reduce the likelihood that a DN mechanism of pathogenesis will be observed, since the proportion of homogeneous wild-type complex will be greater.

Importantly, the dissociation constant of the complex also plays a role in the final 'mixing' with other alleles once diffused away from the polyribosome. For example, the p53 homotetramer, was found to dimerize cotranslationally [68], which ensures that the complex is unlikely to form mixed primary dimers in the protein's short lifetime, promoting a better mixing strategy in the case of DN

Figure 3



Cotranslational assembly of a homomer encoded by a heterozygous allele affects the stoichiometry of assembled complexes and can influence the dominant-negative mechanism of molecular inheritance. If wild-type and mutant subunits associate randomly, the distribution of stoichiometries in **(a)** will be seen, and only 1/16 (6.25%) complexes will be fully wild type. If assembly is completely co- or peri-translational, then the assembles complexes will contain either **(b)** all wild-type or **(c)** all mutant subunits. Finally, if the second assembly step (dimerization of dimers) is not obligately cotranslational, or there is a conformational equilibrium between tetramers and dimers, then the stoichiometries of assembled complexes can be within the ranges shown in **(d)**.

mutations. Some of p53 mutations indeed behave in a DN fashion: mostly structural mutations that can enhance aggregation of wild type that co-exists in the same tetrameric complex. However, the deleterious effect of many mutations can in fact be diluted by the wild type [69•], which may explain why some tumours discard the wild-type allele [70].

Concluding remark

Assembly of protein complexes often occurs very close to the site of translation. This is due to effects of cellular crowding, which limits diffusion, and significantly reduces the probability of lowly expressed subunits finding their binding partners outside of the high local concentrations surrounding the ribosome. Moreover, such an assembly limits the time of uncovered hydrophobic interfaces that makes the unassembled subunits very sensitive to aggregation. Peripheral assembly will also determine the composition of the complex, considering the presence of disease forming alleles. Nevertheless, cotranslational assembly can also carry a heavy cost, namely through the formation of aggregates, whether non-specific or amyloid. As such, the cell must strike a balance between rapid assembly near the ribosome and avoidance of aggregation that ensures the stability of the polypeptide's tertiary and quaternary structure.

To support these ideas, and to further understand the role of cotranslational assembly in normal biological function, as well as its potential implications mitigating the DN effect in inherited and *de novo* genetic disorders, there is a need for new tools and much more experimental characterization cotranslational processes. For example, NMR [71,72], cryoelectron microscopy [42••] and proteomics [40••] have shown great promise, and are likely to continue to do so in coming years.

Conflict of interest

Nothing declared.

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